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DNA Hypermethylation in Sodium Butyrate-treated WI-38 Fibroblasts*

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Sodium butyrate is very often used to alter gene expression in cultured cells. In this study, we examined the effects of this compound on various cellular events in WI-38 human embryonic lung fibroblasts in culture. During a 16–20-h treatment at sodium butyrate concentrations of between 5 and 20 mM, no adverse effects on cell morphology were observed. However, cell division and DNA synthesis were reversibly inhibited, the latter by 85, 80, and 70% at sodium butyrate concentrations of 5, 10, and 20 mM, respectively. Although overall protein synthetic activity was not significantly affected, RNA synthesis decreased to 76% of the control values at a sodium butyrate concentration of 5 mM. Butyrate treatment also caused hypermethylation of DNA cytosines as determined by differential digestion by *MspI/HpaII* restriction endonucleases and by high performance liquid chromatography analysis of the DNA. The 5-methylcytosine content of the DNA in untreated WI-38 fibroblasts was 2.94 ± 0.46% of total cytosine residues, while in cultures treated with 5, 10, and 20 mM sodium butyrate, these values were 5.76 ± 0.28, 5.91 ± 0.37, and 6.8 ± 0.44%, respectively. An interesting feature is that this hypermethylation occurred in DNA which was synthesized in the presence of sodium butyrate (newly synthesized) as well as in DNA which had been synthesized before butyrate administration (pre-existing DNA). The hypermethylated state was conserved only in the former situation, since the methylcytosines were rapidly lost in the subsequent generation in the latter case. It would therefore appear that methylcytosines are maintained after cell replication only if they are generated on newly synthesized DNA.

The four-carbon fatty acid, sodium butyrate, has a wide variety of morphological and biochemical effects on cells in culture (1, 2). Some of the early studies on the effects of sodium butyrate showed that it inhibited cell growth, probably by arresting cells in the G1 phase of the cell cycle (3, 4). This G1 arrest appears to be a specific inhibition at the G1 to S phase transition point, but may also be indirect since butyrate affects a large number of cellular processes and enzyme activities. Effects such as inhibition of DNA replication (5–7) and stimulation of DNA repair synthesis in ultraviolet-irradiated fibroblasts have also been shown to occur (8, 9).

The best studied effect of sodium butyrate is perhaps the reversible hyperacetylation of histones H3 and H4 and, to a lesser extent, that of the histones H2A and H2B (10–13), resulting in increased sensitivity to micrococcal nuclease (14) and increased transcriptional activity (15, 16). Hyperacetylation is due to inhibition of the deacetylase and not to stimulation of the acetylase. Hyperphosphorylation of histone H2A (4, 5), as well as the high mobility group proteins HMG14 and -17 (17), has also been observed. Other effects include inhibition of protein methylation (5), diminished RNA polymerase activity due to a decreased concentration of RNA polymerase molecules (18), and stimulation of ADP-ribose polymerase activity. Thus, sodium butyrate affects the activity of several nuclear enzymes and it is not yet clear whether these multiple effects are due to a single molecular event or whether butyrate acts at various levels.

Understanding the mode of action of sodium butyrate is complicated even further by the fact that individual genes appear to be affected differently in different cell types. The studies of Reeves and Cserjesi (19) and Leibovitch *et al.* (20) have shown that sodium butyrate induces new gene expression in Friend erythroleukemia cells. Analysis of hybridization kinetics showed that as many as 3000 new genes may be transcribed. Examples of specific genes include globin gene expression in murine erythroleukemia cells (21) and induction of oncodevelopmental proteins such as alkaline phosphatase, chorionic gonadotropin, and β-glycoprotein in a cervical carcinoma cell line (22). A very interesting feature of gene induction by butyrate is its synergistic behavior when used in combination with other inducers; for example, the induction of β-adrenergic receptors in HeLa cells (23) and fetal hemoglobin in adult chickens (24) after 5-azacytidine administration.

The mode of action of sodium butyrate in gene activation or repression is presently not fully understood, but it is known that induction of globin gene expression with inducers such as dimethyl sulfoxide and ethionine results in hypermethylation of the globin genes in a murine erythroleukemia cell line (25–27). Although DNA hypomethylation is generally associated with gene activation (28–30), genomic rearrangement (31) could play a major role in gene induction.

Most of the above mentioned studies were performed on transformed cells and it is possible that some of the effects observed are peculiar to "immortal cells." In this study, we have examined the effects of sodium butyrate on the "mortal" fibroblast cell line, WI-38, and have found it to cause hypermethylation of cytosine residues in the total DNA. Furthermore, only those sites which are hypermethylated in the newly synthesized DNA are maintained after the removal of butyrate, while hypermethylation occurring on "old" or pre-existing

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DNA strands does not persist once the stimulus has been removed.

MATERIALS AND METHODS

Cell Culture—WI-38 fibroblasts were obtained from ATCC (ATCC CL-75). Cells were maintained in Eagle's Basal Medium containing 10% fetal calf serum and 100 μg/ml penicillin and 100 units/ml streptomycin. At confluence, the cells were split at a ratio of 1:2. Cells were trypsinized with 0.05% trypsin in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline containing 10 mM EDTA and counted in a Coulter Counter (Coulter Electronics).

DNA, RNA, and Protein Synthesis—Cells were seeded at 5×10^4/7-cm² dish. Twenty h later, [^{3}H]thymidine, [^{3}H]uridine, or [^{3}H]leucine (all from Amersham, at 40 Ci/mmol, 43 Ci/mmol, and 50 Ci/mmol, respectively) was added at 1 μCi/ml in the presence of the specified concentrations of sodium butyrate. After the indicated time period, the cells were trypsinized and an aliquot was counted. Trichloroacetic acid was added to the balance of the cells to a final concentration of 10%. After 1 h on ice, the samples were filtered onto Whatman GF/C filters. When the cells were labeled with [^{3}H]uridine, a 10 μM concentration each of deoxycytidine and thymidine was added in order to reduce incorporation of the label into DNA via the salvage pathways.

DNA-Agarose Gels—Cells were grown in 150-cm² flasks and treated with the indicated concentrations of sodium butyrate, and the DNA was isolated as previously described (32). Briefly, cells were lysed in 1% sodium dodecyl sulfate, 1 mM EDTA and incubated with 100 μg/ml Proteinase K for 4 h at 50 °C. Samples were extracted with an equal volume of phenol saturated with TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA), until no protein interphase was visible. After two extractions with chloroform: isoamyl alcohol (24:1), the DNA was precipitated by the addition of 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethanol at −20 °C for 1 h. The DNA pellet was washed three times in 70% ethanol and dissolved in TE buffer. DNA was digested for 2 h with 5 units of *Hpa*II or *Msp*I per μg of DNA as described by the manufacturers (New England Biolabs or Boehringer Mannheim). Samples were electrophoresed on 1% horizontal agarose gels and stained with ethidium bromide. The negatives were scanned in a Cliniscan densitometer (Helena, Beaumont, TX).

High Performance Liquid Chromatography Analysis—For 5-methylcytosine analysis, the cells were grown in 60-cm² dishes until treated with the various concentrations of sodium butyrate. Cells were either prelabeled for 3 days with [6-^{3}H]uridine, rinsed, and treated with butyrate, or treated simultaneously with [6-^{3}H]uridine and butyrate. In both cases, the butyrate treatment was for 16–20 h. After treatment, the medium was removed and the cell layer was rinsed with phosphate-buffered saline and lysed in 0.5% SDS, 0.3 N NaOH before being incubated at 37 °C for 16 h (33). The incubate was neutralized by the addition of Tris-HCl, pH 7.5, to a final concentration of 50 mM and HCl to 0.3 N. Proteins were digested for a further 24 h by the addition of Proteinase K to a final concentration of 100 μg/ml. The DNA was precipitated by the addition of an equal volume of 10% trichloroacetic acid and left at 4 °C for 1 h. The DNA was pelleted, rinsed in 70% ethanol, and hydrolyzed in 88% formic acid at 180 °C for 25 min in sealed capillary tubes. The formic acid was evaporated and the hydrolysate was resuspended in 0.1 N HCl. Analysis for 5-methylcytosine content was performed by high performance liquid chromatography on a Beckman Ultracil Cx column. The percentage of cytosines (C) that were converted to 5-methylcytosine (5-mC) was calculated as follows:

$$\% \text{ 5mC} = \frac{\text{dpm in 5-mC}}{\text{dpm in 5-mC + C}} \times 100$$

In the dual-labeling experiments, cells were treated with [^{14}C]uridine for 3 days prior to butyrate treatment, followed by [6-^{3}H]uridine during or after treatment.

RESULTS

Cell Growth—Sodium butyrate addition inhibited cell division in WI-38 fibroblasts, even at concentrations of 5 mM (Fig. 1). Determination of cell viability by trypan blue exclusion, on the other hand, showed that this property was not affected by either sodium butyrate or sodium chloride. Butyrate inhibition of cell growth at concentrations as high as 50 mM (data not shown) was such that the cells were still viable and able to resume growth after butyrate removal.

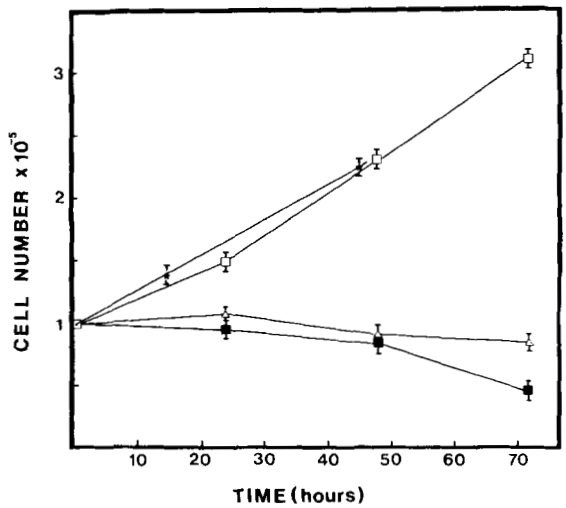

FIG. 1. **Cell growth in the presence of increasing concentrations of sodium butyrate.** Cells were seeded at 10^5 cells/30-mm diameter dish. Sixteen h later, the medium was replaced with fresh medium containing sodium butyrate, at either 5 mM (△——△) or 10 mM (■——■). The effect of sodium ions was monitored by treating the cells with 10 mM NaCl (★——★). Cells were trypsinized and counted as described under "Materials and Methods." □——□, control cells in the absence of the two salts. *Bars* indicate standard deviations.

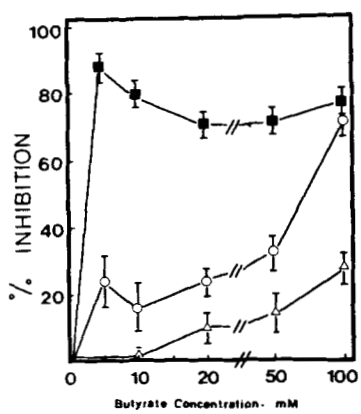

FIG. 2. **Measurement of DNA (■——■), RNA (○——○), and protein (△——△) synthesis in WI-38 fibroblasts during 16–20 h treatment periods at various concentrations of sodium butyrate.** DNA synthesis was measured by the incorporation of [^{3}H]thymidine into trichloroacetic acid-precipitable material, while RNA and protein synthesis were monitored by incorporation of [^{3}H]uridine and [^{3}H]leucine into trichloroacetic acid-precipitable material, respectively (see "Materials and Methods"). *Bars* indicate standard deviations.

DNA, RNA, and Protein Synthesis—Sodium butyrate has previously been shown to inhibit replicative DNA synthesis (5–7). Although DNA synthesis in WI-38 cells was inhibited by approximately 85% at 5 mM butyrate, RNA synthesis was inhibited by only 20% and protein synthesis was unaffected (Fig. 2). Maximum inhibition of DNA synthesis (85%) occurred at 5 mM butyrate, while the inhibition of RNA synthesis did not display this kind of dose response. Protein synthesis was minimally inhibited at 5 mM butyrate; it is therefore improbable that inhibition of DNA synthesis was due to inhibition of protein synthesis.

DNA Methylation—Using the digestion patterns obtained with the *Hpa*II/*Msp*I restriction endonuclease pair, we ex-

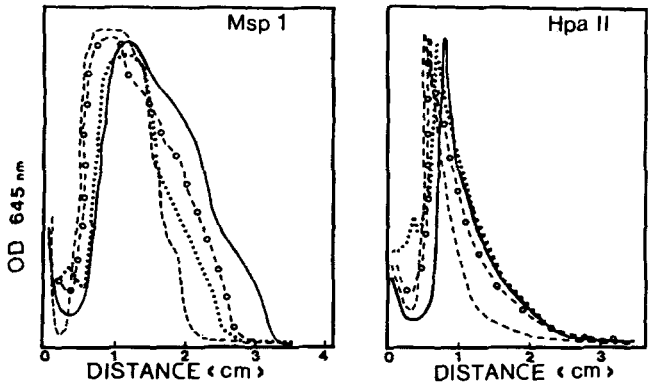

FIG. 3. **Methylation analysis of DNA from control and butyrate-treated cells by digestion with either *Msp*I or *Hpa*II.** DNA was digested at 5 units of enzyme/μg of DNA, electrophoresed on a 1% agarose gel, and stained with ethidium bromide. The negatives were scanned with a Cliniscan densitometer. ——, untreated; - - -, 5 mM; · · · ·, 10 mM; O——O, 20 mM sodium butyrate.

amined the extent of cytosine hypermethylation, if any, after butyrate treatment of WI-38 cells. Fig. 3 shows a densitometric scan of DNA samples isolated from cells either untreated or given 5, 10, or 20 mM sodium butyrate and subjected to electrophoresis on 1% agarose gels after digestion with either *Hpa*II or *Msp*I. Butyrate treatment of cells caused a shift toward the high molecular weight region after restriction endonuclease digestion and this shift occurred in both *Hpa*II- and *Msp*I-digested samples, showing that hypermethylation occurred at both cytosines in the recognition sequence CCGG. This shows that CpC methylation was induced together with that of CpG sequences. The molecular weight shift was greater in the *Msp*I-digested samples, indicating that significant CpC methylation probably occurred even at 5 mM butyrate. Higher levels of methylation were observed at 5 mM than at either 10 or 20 mM butyrate.

In order to determine the extent of DNA hypermethylation, the cells were treated with [6-^{3}H]uridine as previously described (33) and the conversion of cytosine to 5-methylcytosine was determined by high performance liquid chromatography analysis of the bases after formic acid hydrolysis. There was a large increase in the 5-methylcytosine content of DNA from 2.94% in control cells to 5.76% in 5 mM butyrate-treated cells, followed by a more gradual increase to 6.8% at 20 mM butyrate (Fig. 4). At butyrate concentrations of 50–75 mM (where NaCl showed no adverse effects), the 5-methylcytosine levels declined back to control values (data not shown). Since the DNA was labeled before butyrate administration, analysis of ^{3}H-labeled 5-methylcytosine in this case would indicate methylation of pre-existing DNA strands. Similar increases in 5-methylcytosine levels were obtained in cells treated with butyrate and [6-^{3}H]uridine concurrently (Fig. 4). In this case, however, only the DNA strands synthesized during the butyrate treatment period were labeled and methylation of these newly synthesized DNA strands was assessed. It was thus evident that both pre-existing and newly synthesized DNA strands were hypermethylated as a result of sodium butyrate treatment, with hypermethylation being greater in the case of pre-existing DNA strands. In fact, pre-existing DNA strand methylation was increased to 5.76% even at 5 mM butyrate, while newly synthesized DNA strands showed no significant increase in 5-methylcytosine content at this low concentration of butyrate.

Maintenance of the Hypermethylated State—In order to determine whether the increased methylation status induced by sodium butyrate was stable during subsequent cell division, cells were analyzed for the 5-methylcytosine content of their DNA 24 h after butyrate treatment, when a single round of replication had occurred in the asynchronous cultures. DNA was labeled with [6-^{3}H]uridine for 3 days, followed by sodium butyrate treatment for 16 h in order to examine methylation of pre-existing DNA strands; alternatively, labeling was done overnight in the presence of butyrate for analysis of newly synthesized DNA strands. Since the lowest concentration of sodium butyrate at which hypermethylation of both pre-existing and newly synthesized DNA strands occurred was 10 mM, the experiments were done at this concentration of

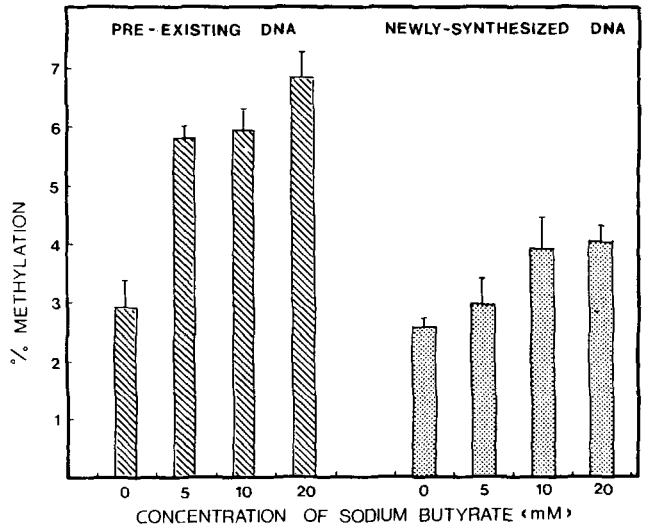

FIG. 4. **DNA hypermethylation analysis by high performance liquid chromatography.** Cells were either prelabeled with [6-^{3}H]uridine followed by butyrate treatment (pre-existing DNA strands) or labeled concurrently with butyrate treatment (newly synthesized DNA strands). After 16–20 h in the presence of butyrate, DNA was isolated and the 5-methylcytosine content was determined as described under "Materials and Methods" (n = 12). *Bars* indicate standard deviations.

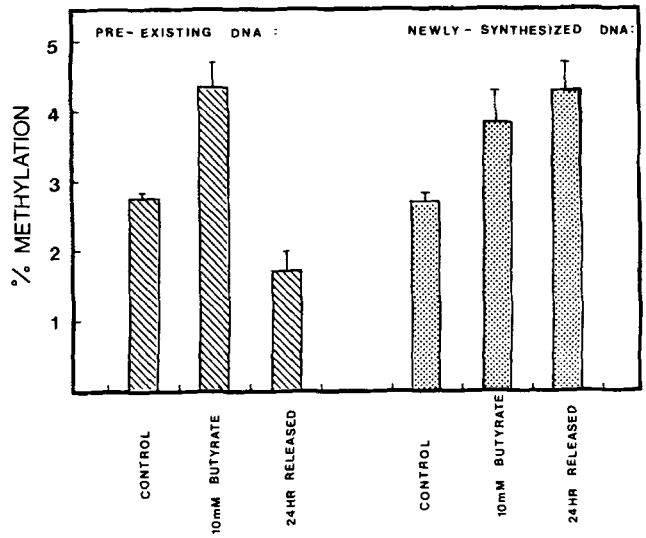

FIG. 5. **Stability of the hypermethylation status in butyrate-treated cells.** Cells were either prelabeled (pre-existing DNA strands) or labeled concurrently (newly synthesized DNA strands) with [6-^{3}H]uridine during butyrate treatment as described in the legend to Fig. 4. Half the flasks were then removed and changed to fresh medium for 24 h, while the other set was analyzed for 5-methylcytosine content immediately. Number of determinations per experiment = 8. *Bars* indicate standard deviations.

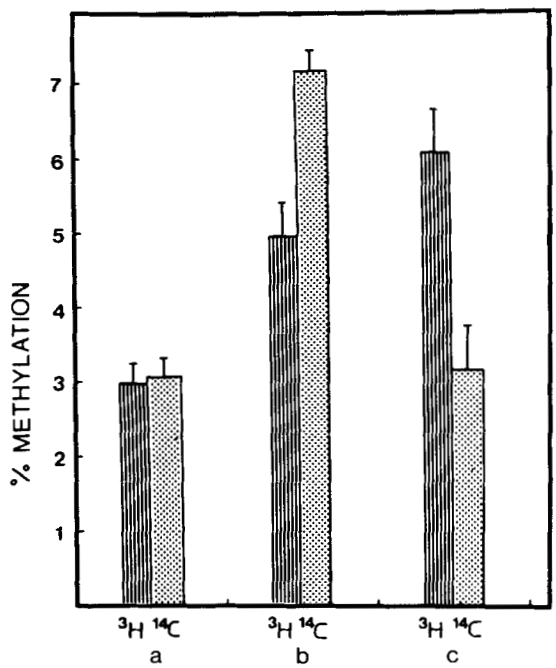

FIG. 6. **Dual label analysis of the stability of DNA hypermethylation.** WI-38 cells were prelabeled with [^{14}C]uridine for 3 days. After extensive washing of the cell layer, medium containing [6-^{3}H]uridine plus 10 mM sodium butyrate was added. The 5-methylcytosine content of the DNA samples was determined in the following cases. *a*, 16 h after the addition of [6-^{3}H]uridine to ^{14}C-prelabeled cells without butyrate addition; *b*, as in *a* above, except that 10 mM sodium butyrate was added together with the [6-^{3}H]uridine for the 16-h pulse; *c*, as in *b* above, except that the cells were allowed to grow with no additions for an additional 24 h after the 16-h pulse in the presence of 10 mM butyrate. *Bars* indicate standard deviations.

butyrate. To our surprise, only those additional 5-methylcytosine residues which were added to the newly synthesized DNA were conserved (Fig. 5) while those occurring on the pre-existing DNA strands were not conserved. Because the above determinations had been made in separate cultures, dual label experiments were also performed in order to confirm the above findings with respect to the maintenance of the methylation status of pre-existing and newly synthesized DNA strands in the same cultures. Cells were prelabeled with [^{14}C]uridine for 3 days, followed by [^{3}H]uridine plus sodium butyrate for 16 h. This design ruled out variations from dish to dish or between experiments. The results (Fig. 6) were in exact agreement with those shown in Fig. 5: 5-[^{14}C]methylcytosines present on pre-existing DNA strands were not conserved, while the 5-[^{3}H]methylcytosines on the newly synthesized DNA strands were retained in daughter cells. This dual-labeling experiment incidently also confirmed the finding that the extent of hypermethylation was much greater in the pre-existing DNA strands than in the newly synthesized DNA strands.

DISCUSSION

Our results are in general agreement with those of other workers on the effects of sodium butyrate on cell growth and DNA synthesis in chicken fibroblasts and HeLa cells (5–7). WI-38 human embryonic lung fibroblasts showed a reversible growth arrest at butyrate concentrations below 20 mM, although the cells could tolerate higher concentrations. By contrast, simian virus 40 (SV40)-transformed WI-38 fibroblasts were much more sensitive to sodium butyrate, even at concentrations as low as 5 and 10 mM (35).

Analysis of macromolecular synthesis showed that although DNA synthesis was drastically affected by butyrate, RNA and protein synthesis were much less affected. In fact, protein synthesis was only marginally affected, with significant inhibition occurring only at concentrations of 20 mM butyrate and higher. Therefore, a general impediment in the synthesis of proteins did not cause the inhibition of DNA synthesis and cell growth. Several studies have shown that sodium butyrate does not inhibit major mRNA or protein synthesis, but that new gene products may be induced (for review see Ref. 2). Whether a gene is to be activated or inhibited by sodium butyrate is probably dependent on a specific precondition of the chromatin and/or the cell type being treated. It is therefore conceivable that only those genes having the potential to be activated will be induced by sodium butyrate (or any other inducer). It is also possible that a specific pre-existing chromatin structure and/or chromosomal factors guide enzymes such as DNA methyltransferases to certain restricted groups or sites on the DNA, resulting in the observed effects. That is, inducers themselves are nonspecific, but gene-specific activation is due to the particular conformation of the genes in chromatin.

One of the significant findings of our study was that butyrate caused hypermethylation of cytosine residues in the DNA of butyrate-treated cells. Several studies have indicated that methylation *per se* may not be the only controlling mechanism of gene activity. We have previously shown that loss of type I procollagen gene expression in SV40-transformed cells is associated with hypermethylation of these genes (32), but that demethylation of these sites with the cytosine analogue 5-azacytidine (34) does not cause expression of the type I procollagen genes (36). It is also possible that a subset of 5-methylcytosine residues is important in the regulation of gene expression, since McKeon *et al.* (37) have shown that methylation of certain cytosine residues has no correlation with activity of the type I procollagen genes. In this case, our study does not prove that butyrate "switches off" 20% of the genes in WI-38 cells as it is also possible that the overall transcriptional activity is only 80% efficient in the presence of butyrate.

Previous studies have shown that DNA methylation is tightly coupled to DNA replication (38, 39) and the fact that most of the 5-methylcytosines occur in the dinucleotide CpG provides a mechanism for the heritable transmission of methylation patterns (30). Our results show that butyrate treatment was not only associated with the methylation of cytosines at CpG sequences, but also at CpC sites (Fig. 3). This restriction endonuclease assay showed that higher levels of CCGG methylation were obtained at 5 mM than at 10 or 20 mM butyrate. However, high performance liquid chromatography analysis (Fig. 4) showed that the 5-methylcytosine content began to plateau at 5 mM, such that no significant further change occurred at either 10 or 20 mM butyrate. The only possible explanation for this discrepancy is that more erratic methylation (*i.e.* at non-CpG sites) occurred at the higher butyrate concentrations and therefore could not be detected by digestion with *Msp*I. A second and unexpected result was that only some of these hypermethylated sites were heritable, namely those in the newly synthesized strands (*i.e.* DNA synthesized in the presence of butyrate) while pre-existing DNA strand hypermethylation was not heritable. This discrimination may be due to more extensive methylation at non-CpG sites in the pre-existing DNA strands, *i.e.* at CpA, CpC, and CpT sequences, while most of the hypermethylation induced on the newly synthesized DNA may be at CpG sites. The finding that hypermethylation of pre-existing

DNA strands occurred with a much higher frequency and at lower butyrate concentrations also suggests that more CpX methylation occurred in the pre-existing DNA strands. These findings were consistent when dual-labeling experiments for pre-existing and newly synthesized DNA strands in the same cultures were employed.

The ability of sodium butyrate to induce hypermethylation of DNA and the apparent differential heritability of this hypermethylated state, with respect to pre-existing and newly synthesized DNA strands, add further important effects of sodium butyrate to an already lengthy list (2). It is possible that methylation levels are "fine tuned" by a demethylating enzyme and that butyrate acts by inhibiting this demethylase rather than by stimulating the methyltransferases or by making more sites on the chromatin accessible to such enzymes.

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